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Survivin and the inner centromere protein INCENP show similar cell-cycle localization and gene knockout phenotype.
Uren A G; Wong L; Pakusch M; Fowler K J; Burrows F J; Vaux D L; Choo K H
The Walter and Eliza Hall Institute of Medical Research, Post Office Royal Melbourne Hospital, 3050,., Victoria, Australia.
Current biology - CB (ENGLAND) Nov 2 2000, 10 (21) p1319-28, ISSN 0960-9822 Journal Code: 9107782

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INCENP is required for proper targeting of Survivin to the centromeres and the anaphase spindle during mitosis.
Wheatley S P; Carvalho A; Vagnarelli P; Earnshaw W C
Chromosome Structure Group, Institute of Cell and Molecular Biology, Swann Building, University of Edinburgh, King's Buildings, Mayfield Road, EH9 3JR, Edinburgh, United Kingdom.
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3) ####14149991 PMID: 9850056
IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas (CD95), Bax, caspases, and anticancer drugs.
Tamm I; Wang Y; Sausville E; Scudiero D A; Vigna N; Oltersdorf T; Reed J C
The Burnham Institute, La Jolla, California 92037, USA.
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Crystal structure and mutational analysis of the Saccharomyces cerevisiae cell cycle regulatory protein Cks1: implications for domain swapping, anion binding and protein interactions.
Bourne Y; Watson M H; Arvai A S; Bernstein S L; Reed S I; Tainer J A
Centre National de la Recherche Scientifique, Marseille, France. yves@afmb.cnrs-mrs.fr
Structure with Folding & design (ENGLAND) Aug 15 2000, 8 (8) p841-50 , ISSN 0969-2126 Journal Code: 100889329
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5) tle: P35 IS A NEURAL-SPECIFIC REGULATORY SUBUNIT OF CYCLIN-DEPENDENT KINASE-5 (Abstract Available)
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Journal: NATURE, 1994, V371, N6496 (SEP 29), P419-423

6) $$$ Crystal structure and mutational analysis of the human CDK2 kinase complex with cell cycle-regulatory protein CksHs1.
Bourne Y; Watson M H; Hickey M J; Holmes W; Rocque W; Reed S I; Tainer J A

Survivin and the inner centromere protein INCENP show similar cell-cycle localization and gene knockout phenotype

Anthony G. Uren*†, Lee Wong†‡, Miha Pakusch*, Kerry J. Fowler‡, Francis J. Burrows§, David L. Vaux* and K.H. Andy Choo‡

Background: Survivin is a mammalian protein that carries a motif typical of the inhibitor of apoptosis (IAP) proteins, first identified in baculoviruses. Although baculoviral IAP proteins regulate cell death, the yeast Survivin homolog Bir1 is involved in cell division. To determine the function of Survivin in mammals, we analyzed the pattern of localization of Survivin protein during the cell cycle, and deleted its gene by homologous recombination in mice.

Results: In human cells, Survivin appeared first on centromeres bound to a novel para-polar axis during prophase/metaphase, relocated to the spindle midzone during anaphase/telophase, and disappeared at the end of telophase. In the mouse, Survivin was required for mitosis during development. Null embryos showed disrupted microtubule formation, became polyploid, and failed to survive beyond 4.5 days post coitum. This phenotype, and the cell-cycle localization of Survivin, resembled closely those of INCENP. Because the yeast homolog of INCENP, Sli15, regulates the Aurora kinase homolog Ipl1p, and the yeast Survivin homolog Bir1 binds to Ndc10p, a substrate of Ipl1p, yeast Survivin, INCENP and Aurora homologs function in concert during cell division.

Conclusions: In vertebrates, Survivin and INCENP have related roles in mitosis, coordinating events such as microtubule organization, cleavage-furrow formation and cytokinesis. Like their yeast homologs Bir1 and Sli15, they may also act together with the Aurora kinase.

Addresses: *The Walter and Eliza Hall Institute of Medical Research, Post Office Royal Melbourne Hospital, Victoria 3050, Australia. ‡The Murdoch Childrens Research Institute, Royal Childrens Hospital, Flemington Road, Parkville, Victoria, 3052, Australia. §Idun Pharmaceuticals, 11085 North Torrey Pines Road, La Jolla, California 92037, USA.

Correspondence: David L. Vaux
E-mail: vaux@wehi.edu.au

†A.G.U. and L.W. contributed equally to this work.

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Background

Inhibitor of apoptosis (IAP) proteins were first identified as baculoviral products that could inhibit the defensive apoptotic response of infected insect cells. Subsequently, a number of cellular IAP homologs were found in diverse organisms including vertebrates, insects, nematodes and yeasts. All of these proteins bear one to three zinc-binding motifs termed baculoviral IAP repeats (BIRs) [1–3]. Biochemical and genetic evidence indicates that some IAPs—such as MIHA/XIAP/hILP, MIHB/c-IAP-1/hIAP2, MIHC/cIAP-2/hIAP1 [4–7] and *Drosophila* DIAP1 [8,9]—are able to inhibit caspase-mediated apoptosis directly or indirectly (reviewed in [10]). In contrast, IAPs from *Caenorhabditis elegans* and the yeasts *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* do not appear to be caspase inhibitors, but seem to function during cell division. For example, *C. elegans* zygotes lacking BIR-1 fail to undergo cytokinesis, *S. pombe bir1* mutants have defects in spindle elongation, and Bir1p from *S. cerevisiae* associates with kinetochore proteins [11–15].

The kinetochore is a DNA–protein complex that assembles on the centromere and is required for attachment of microtubules during mitosis. Some mammalian centromere-interacting proteins (such as CENP-A, CENP-B and CENP-C) associate constitutively, whereas others (such as CENP-E, CENP-F and the inner centromere protein INCENP), collectively known as chromosome passenger proteins, localize transiently to the centromere during specific stages of the cell cycle (reviewed in [16]). As the cell cycle progresses into metaphase, INCENP becomes concentrated at centromeres. During the metaphase–anaphase transition, INCENP remains confined to the equator while the sister chromatids migrate to the poles. During telophase, it is located in the midbodies at the intercellular bridge, and is degraded after cytokinesis [17,18]. The timing of expression and distribution of INCENP resembles that of Aurora1, a member of the Aurora/Ipl1p family of serine threonine kinases ([19], reviewed in [20]). Furthermore, overexpression of kinase-inactivated Aurora1 disrupts cleavage-furrow formation, resulting in a failure of cytokinesis similar to that caused by deletion or expression of a dominant-negative INCENP [21–23]. Therefore, INCENP and Aurora1 may have related roles during mitosis.

Survivin is a mammalian protein that has a single BIR [24]. Structurally, it resembles more closely the BIR-containing proteins from yeasts and *C. elegans* [2] than it does the IAPs that control apoptosis. Survivin expression is regulated during the cell cycle [25,26], and inhibition of

Survivin has been associated with cell-cycle defects [27]. Because the BIR-containing proteins from *S. pombe*, *S. cerevisiae* and *C. elegans* all have roles in cell division [11,12,15], Survivin may have a similar function. Here, we raised antibodies against Survivin and analyzed its pattern of expression during the cell cycle. To determine the requirement for Survivin in normal cells, we deleted its gene by homologous recombination in mice. The phenotype of the *survivin* null mouse embryos, and the pattern of Survivin staining, are consistent with a role as a chromosome passenger protein that functions during mitosis.

Results

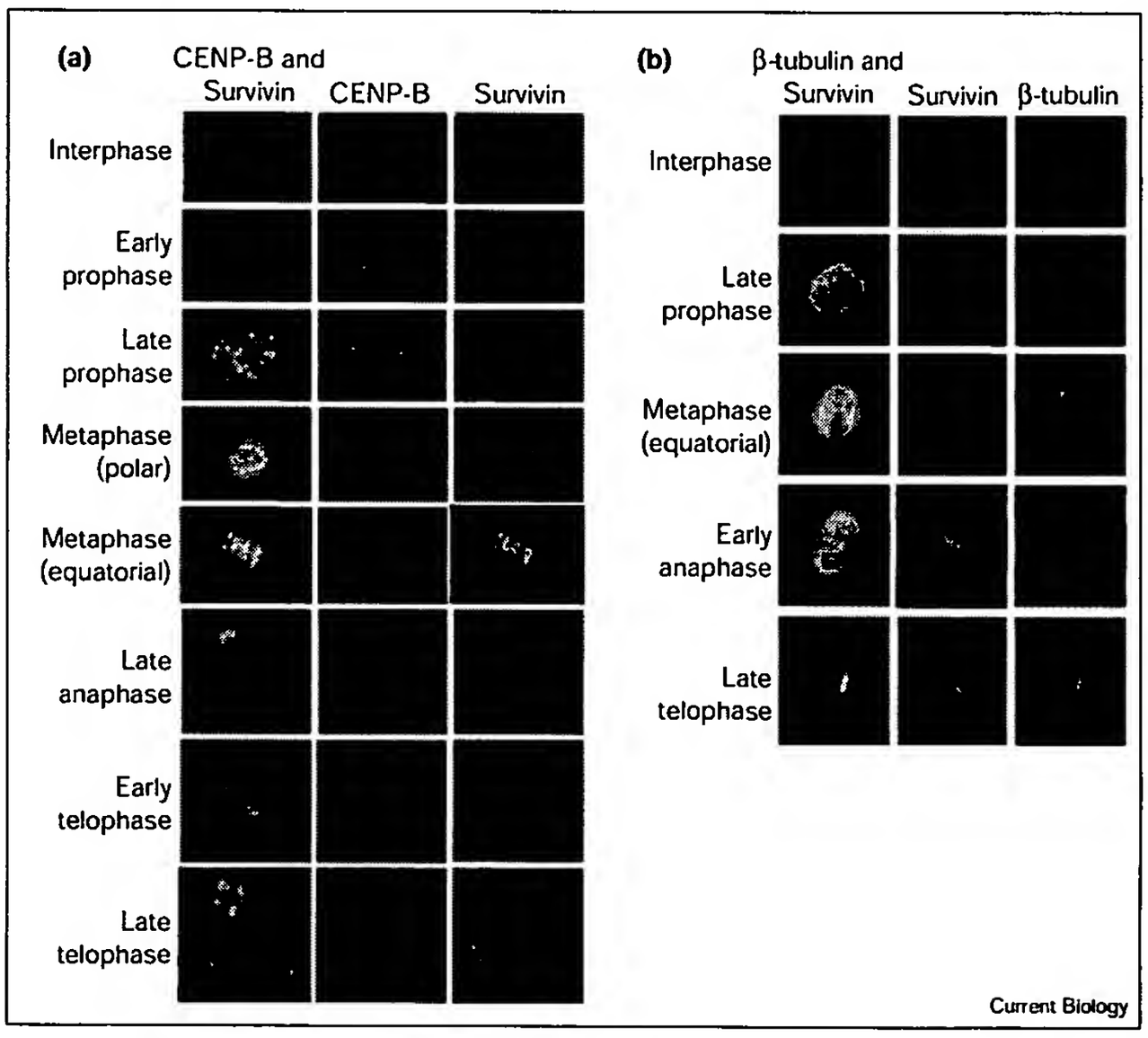
Localization of Survivin during the cell cycle

We determined the distribution of Survivin during the cell cycle using cultured HeLa cells. Initial immunofluorescence staining using the anti-Survivin antibody TO65 revealed strong punctate signals on condensed chromatin that were suggestive of specific association of Survivin with chromosomal structures (data not shown). This prompted us to compare the localization of Survivin with that of the centromeric α-satellite DNA-binding protein CENP-B, using the monoclonal antibody 2D-7. As expected for a constitutive centromere protein, CENP-B was detected as discrete spots on centromeres throughout the cell cycle (Figure 1a). No Survivin was detectable in the interphase cells. A low level was detected at early prophase, during which no specific localization to the centromere was apparent. In late prophase, nuclear staining became intense, with foci of Survivin now colocalizing with CENP-B. As the cells progressed into metaphase, Survivin became prominently concentrated on centromeres, as shown by strong colocalization with CENP-B. At anaphase, while CENP-B moved with the separating sister chromatin masses to the poles, Survivin remained at the midzone where the metaphase plate once was. During telophase, Survivin was found on the midbody between the daughter cells, and after telophase it was degraded.

In view of previous studies indicating the close association of Survivin with the microtubules [26,27], we compared the cell-cycle distribution profiles of Survivin and microtubules, using a monoclonal anti-β-tubulin antibody (Figure 1b). No significant colocalization was observed between interphase and metaphase. During early anaphase, some Survivin began to relocate onto the microtubules at the spindle midzone. By late anaphase and telophase, Survivin colocalized with the concentrated intercellular microtubule bundles that formed the midbody. This distribution profile is characteristic of the chromosome passenger class of proteins.

We were also interested to know whether disrupting the integrity of microtubules had any effect on the localization

Figure 1



Cell-cycle distribution of Survivin, CENP-B and β-tubulin. **(a)** Simultaneous immunofluorescence staining for Survivin (green) and CENP-B (red) in HeLa cells grown on coverslips and analyzed at different cell-cycle stages. Two different views of metaphase cells are shown, one from the pole (presenting a 'rosette' configuration), the other from the side (showing chromosomal congression on the equatorial plate). Left panels, combined CENP-B and Survivin immunofluorescence; the chromatin was stained with 4,6-diamidino-2-phenylindole (DAPI, blue). Middle and right panels, CENP-B and Survivin staining, respectively. **(b)** Simultaneous immunofluorescence staining for Survivin (green) and β-tubulin (red) during different cell-cycle stages of HeLa cells grown on coverslips.

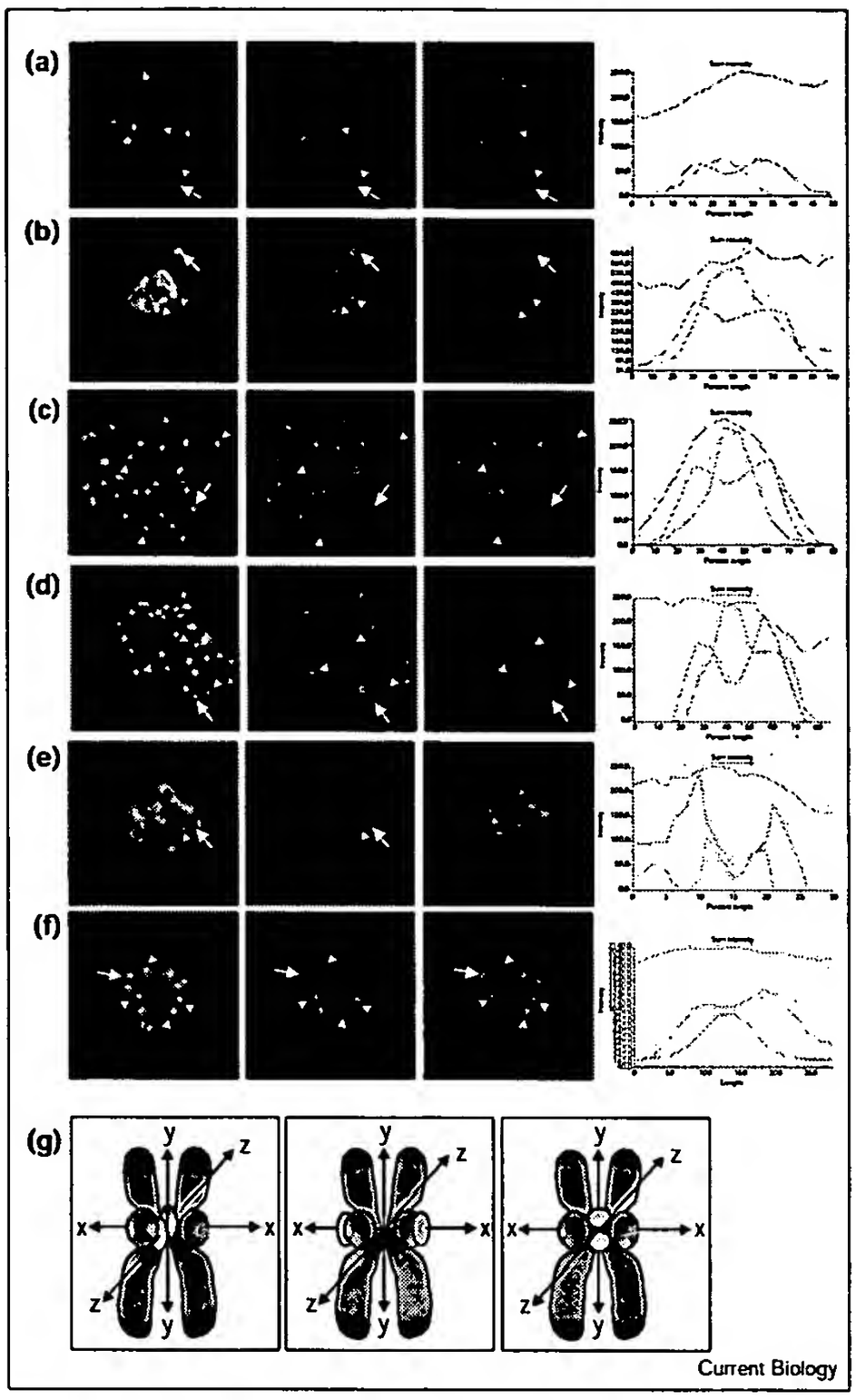


Figure 2

Immunofluorescence analysis of Survivin and various centromere-binding proteins. (a–d) Dual-color immunofluorescence analysis using CREST#6 (green) and anti-Survivin antibody (red). Metaphase chromosomes were prepared from HeLa cells treated with (a) nocodazole, (b,d) taxol, or (c) colcemid. The cells in (a,b) were cultured on coverslips directly, whereas the cells in (c,d) were spun onto a slide by cytocentrifugation (which gave better spreading of metaphases). First column, combined immunofluorescence images in which the chromatin was stained with DAPI (blue). The green and red signals are shown individually in the second and third columns, respectively. Note that CREST#6 and Survivin both bound to the centromere to give doublet signals. Nevertheless, whereas the paired signals for CREST#6 were generally similar in intensity when viewed on the same focal plane, the Survivin doublet signals (some examples shown by arrowheads) were apparent only when viewed on different focal planes (not shown). Fourth column, multi-color scanning of the centromere (arrowed) along the trans-polar axis. 'Length' indicates position along the trans-polar axis, and 'intensity' indicates the fluorescence signal. Both are in arbitrary units. The colors of the different curves correspond to those of the images shown in the first three columns. **(e,f)** Dual-color immunofluorescence analysis using (e) CREST#6 (red) and anti-CENP-E antibody (green), or (f) CREST#6 (red) and anti-INCENP antibody (green). Metaphase chromosomes were prepared from HeLa cells grown on a coverslip in normal culture media. The arrows and arrowheads point to examples of centromeres showing trans-polar binding of the CENP-E signal in (e), or para-polar association of the INCENP signal in (f). The examples indicated by the arrows were used to derive the multi-color scanning profile shown in the fourth column. **(g)** Models for the different subregional topographic distribution of centromere proteins along three possible axes: the x- or trans-polar axis, which connects the midpoints of the two opposite-facing kinetochore discs; the y- or longitudinal axis, which runs parallel to the long axis of the chromosome, between the kinetochore discs; and the z- or para-polar axis, which bisects between the kinetochore discs perpendicular to both the x and y axes. Left panel, alignment of CREST#6 antigen doublet signal at the kinetochore discs (green, CENP-A binding) and inner centromere heterochromatin (red, CENP-B binding) along the trans-polar (x) axis, and the Survivin and INCENP doublet signals (gray) at a midpoint between the sister chromatids along the para-polar (z) axis. Sister chromatids are shown in blue. Middle panel, as in the left panel except yellow signals represent binding of CENP-E and CENP-F to the outer edges of the kinetochore discs or corona regions along a common trans-polar (x) axis. Right panel, previously described binding of INCENP (pink) along the y axis [41] or throughout the heterochromatic region (red) beneath the kinetochore discs (green) along the x axis [42,43] (see Discussion).

of Survivin at the centromere. For this analysis, HeLa cells were cultured in the presence of taxol (a microtubule-stabilizing agent), or nocodazole or colcemid (microtubule-destabilizing agents). We also compared cells that had been grown directly on coverslips with cells that were spread onto slides by cytocentrifugation, a procedure that disrupts microtubules mechanically. In each case, strong colocalization of Survivin with the CREST#6 antibodies (anti-CENP-A and anti-CENP-B antibodies) on metaphase chromosomes was observed (Figure 2a–d). Therefore, binding of Survivin to the centromere did not depend on the integrity of the microtubules.

Survivin binds to the centromere on a different axis to the kinetochore axis

Earlier studies have described the binding patterns of more than 20 different proteins on the metaphase centromere (for example, [28,29]). Although the distance between the paired signals for these proteins on the two chromatids varies slightly depending on whether binding is at the inner centromere region, on the kinetochore, or at the outer kinetochore region, the evenness of the doublet signal intensity indicates that antigen binding occurs along the same axis (designated as the trans-polar or x axis) joining the two kinetochore discs. This pattern was typical for staining with the CREST#6 antiserum, shown in Figure 2a–f.

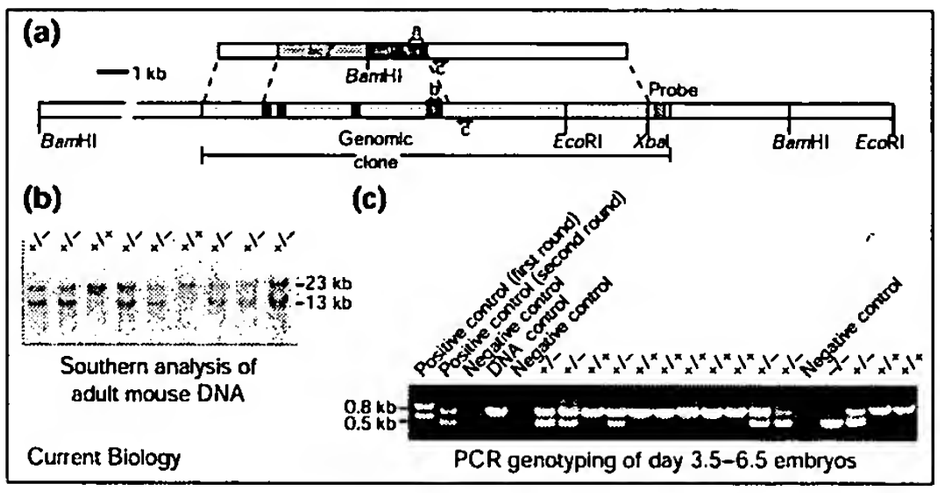
Even though Survivin also bound to the metaphase centromeres to give doublet signals, the two Survivin spots were not in the same focal plane. Furthermore, Survivin was generally present at the midpoint of the CREST#6 doublet signals, as evident from direct immunofluorescence visualization and from multi-color electronic scanning of centromere staining (Figure 2a–d). Survivin was located along an axis (denoted here as the para-polar or z axis) that perpendicularly bisects both the trans-polar x axis

and the longitudinal y axis that runs parallel to the long axis of the chromosome, between the kinetochore discs (see Figure 2g). Similar results were obtained in cells that had not been drug-treated, and in cells grown directly on coverslips or centrifuged onto slides, suggesting that the observed para-polar alignment was not an artifact of microtubule inhibition or cytocentrifugation (Figure 2 and data not shown). For comparison, we examined the staining patterns for three other chromosomal passenger proteins (CENP-E, CENP-F and INCENP). The results indicated that CENP-E bound typically as a doublet of even intensity along the same trans-polar axis as that for the CREST#6 signals, although the distance between the doublet spots for CENP-E was noticeably greater than that for CREST#6, suggesting that CENP-E binding occurred external to the regions occupied by CENP-A and CENP-B (Figure 2e). The results for CENP-F (previously shown to interact with CENP-E [30]) were identical to those for CENP-E (data not shown), whereas INCENP binding was detected along the same para-polar axis as that seen for Survivin (Figure 2f). The relative subregional centromeric distribution profiles for CENP-A/CENP-B, Survivin, INCENP, CENP-E and CENP-F are illustrated in Figure 2g.

Cloning and characterization of the mouse *survivin* locus and deletion of the gene

To determine the requirement for Survivin in mouse embryos, we deleted the gene by homologous recombination. The human and mouse *survivin* transcripts encode proteins of 142 and 140 amino acids, respectively [24,25]. We obtained both human and mouse *survivin* cDNA clones from the IMAGE consortium [31] and sequenced them (accession numbers AF077349 and AF077350). The human *survivin* gene has previously been reported to have significant similarity to the EPR-1 cDNA in its antisense orientation, suggesting a common origin for both loci [32,33]. There is, however, no similarity to the EPR-1 open reading frame or any other protein in the opposite orientation of the mouse *survivin* transcript. Thus, it appeared that deletion of the mouse *survivin* locus should not affect expression of EPR-1, if indeed it exists [34]. A mouse *survivin* cDNA was used to screen a mouse genomic library. Multiple independent lambda phage clones were isolated and mapped by restriction enzyme digestion, and 9 kb of the longest clone was sequenced, spanning the region encoding the whole of the mouse *survivin* transcript (accession number AF077351; Figure 3).

Figure 3



Deletion of the *survivin* gene by homologous recombination. **(a)** The gene targeting construct replaced a genomic fragment bearing all four exons (labeled 1–4) of *survivin* with a cassette encoding β-galactosidase in-frame after the ATG. A probe 3′ to the region recombined was used to type mutated embryonic stem (ES) cells by Southern blot analysis. **(b)** The same probe was hybridized to *Bam*HI-digested tail DNA from adult mice and detected a 23 kb wild-type allele and a 13 kb targeted allele. **(c)** Day 3.5–6.5 embryos from heterozygous intercrosses were typed by PCR using nested primers (denoted a, b and c), which generated a 0.8 kb wild-type product and a 0.5 kb targeted product. The positive controls were tail DNA from heterozygous and wild-type mice, and the negative controls contained no DNA.

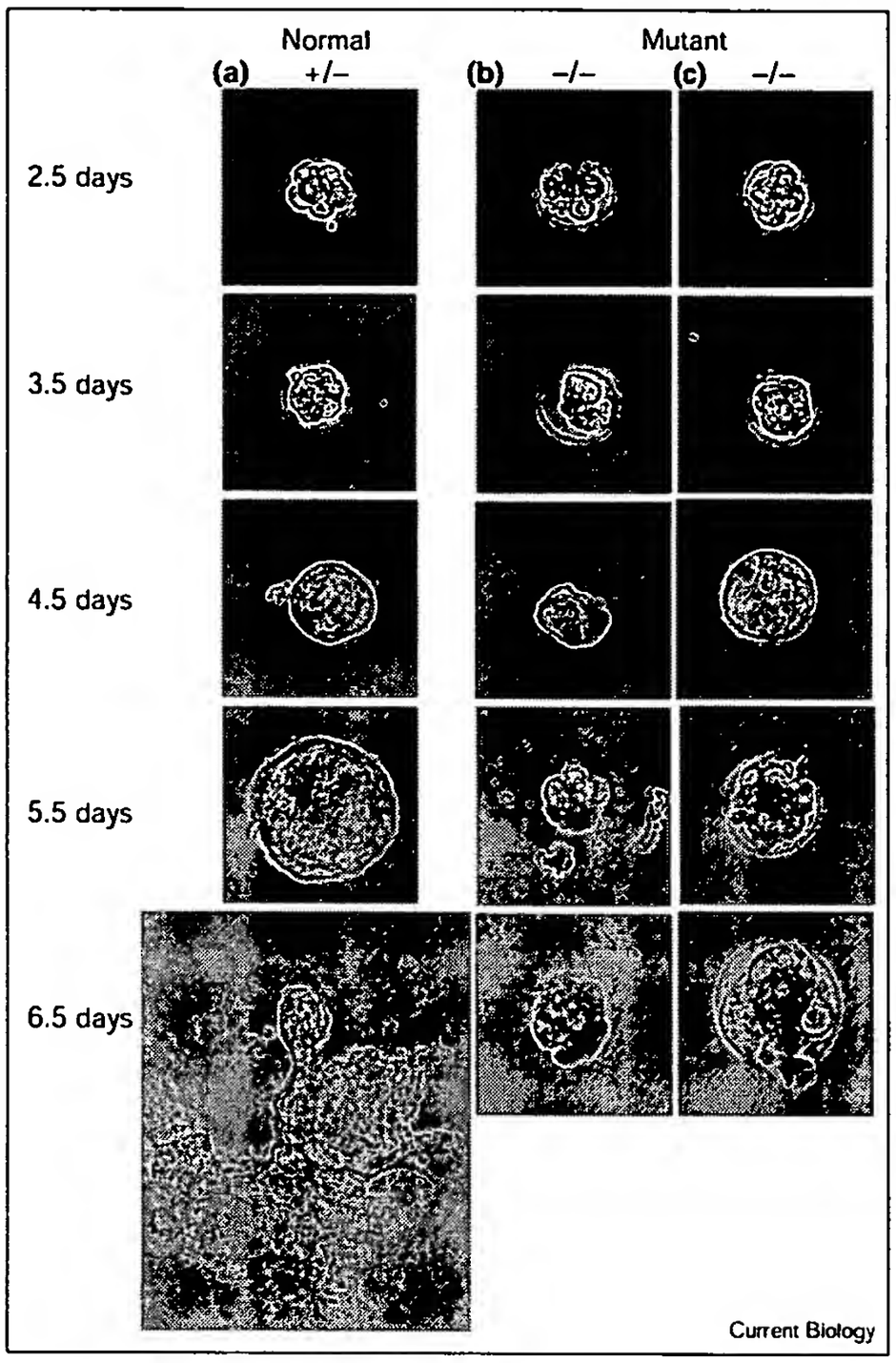
A vector for targeted disruption of *survivin* was designed that replaced all sequences except those encoding the first four amino acids of the *survivin* open reading frame with the *lacZ* gene. The 5′ and 3′ arms of the vector were 2 and 6.5 kb in length, respectively, and the selectable marker was a neomycin cassette flanked by *loxP* sites. This construct was electroporated into the C57Bl/6-derived Bruce 4 ES cell line. Clones with homologous integrations of the vector were identified by Southern hybridization with a probe external to the vector (Figure 3) and used to generate chimeras that passed the mutation through the germ line. Of the first 39 pups born from mating of heterozygous parents, Southern analysis of tail DNA revealed 13 were wild type and 26 were heterozygous. No homozygous *survivin* mutant mice were found, or have been identified to date, demonstrating that loss of the *survivin* gene caused embryonic lethality. The ratio of wild-type to heterozygous mice indicated that loss of one copy of the *survivin* gene was unlikely to cause developmental defects.

Developmental lethality of *survivin* null embryos

Embryos from heterozygous intercrosses were flushed on day 2.5 post coitum and their development observed in culture. Of a total of 96 embryos collected, 73 showed normal development, whereas 23 developed abnormally. When the embryos were collected for genotyping by PCR on day 6.5, a complete correlation between embryos showing a deteriorating phenotype and the *survivin*$^{-/-}$ genotype was observed, indicating that 24% of the total embryos were homozygous mutants. This suggests that there were no significant losses of the *survivin*$^{-/-}$ embryos before day 2.5.

The onset of morphological deterioration was variable amongst the *survivin*$^{-/-}$ embryos. In most of the embryos, degeneration was apparent at day 2.5 (Figure 4b). Of the remaining *survivin*$^{-/-}$ embryos, the morphology was indistinguishable from the *survivin*$^{+/+}$ and *survivin*$^{+/-}$ embryos up to day 4.5, during which blastocysts containing an inner

Figure 4



Morphology of normal and *survivin* null embryos. Embryos were collected at day 2.5 post coitum and cultured *in vitro* up to day 6.5. **(a)** The normal embryo, subsequently confirmed by PCR genotyping to be *survivin*$^{+/-}$, developed an inner cell mass, a blastocoel cavity, and hatched out of the zona pellucida by day 4.5, forming an expanded inner cell mass and surrounding trophoblast cells by day 6.5. The *survivin*$^{-/-}$ embryo in **(b)** showed deterioration of a number of the blastomeres at day 2.5, but managed to continue developing further, albeit aberrantly. The *survivin*$^{-/-}$ embryo in **(c)** showed a seemingly healthy morphology up till 4.5 days. By day 5.5, both *survivin*$^{-/-}$ embryos showed gross cellular degeneration, absence of distinct inner cell mass, blastocoel cavity, or trophoblasts, and the formation of giant cells. Magnification ×200, phase contrast microscopy.

cell mass and blastocoel cavity were formed (Figure 4c). However, by days 5.5 and 6.5, all *survivin*$^{-/-}$ embryos were grossly abnormal and showed deteriorated cell masses and giant cells, whereas the *survivin*$^{+/+}$ and *survivin*$^{+/-}$ embryos had gone on to form a compact inner cell mass and spreading trophoblast cells. Furthermore, the *survivin*$^{-/-}$ embryos never hatched out of their zona pellucida over the study period, whereas hatching occurred around day 4.5 for all of the wild-type and heterozygous embryos.

Table 1

Number of nuclei.

Days	+/+ or +/–	–/–*	*p* value
2.5	11.2 ± 3.2 (*n* = 11)	8.0 ± 2.4 (*n* = 7)	0.051
3.5	40.4 ± 3.3 (*n* = 18)	19.6 ± 6.1 (*n* = 9)	< 0.001
4.5	76.2 ± 9.7 (*n* = 6)	23.25 ± 9.9 (*n* = 8)	< 0.001
5.5	232.5 ± 22.3 (*n* = 6)	13.4 ± 2.7 (*n* = 9)	< 0.001

The number of nuclei were determined in embryos from heterozygous intercrosses at day 2.5 of development *in utero* or following culture (days 3.5–5.5); *n*, number of embryos analyzed. *Micronuclei were not included.

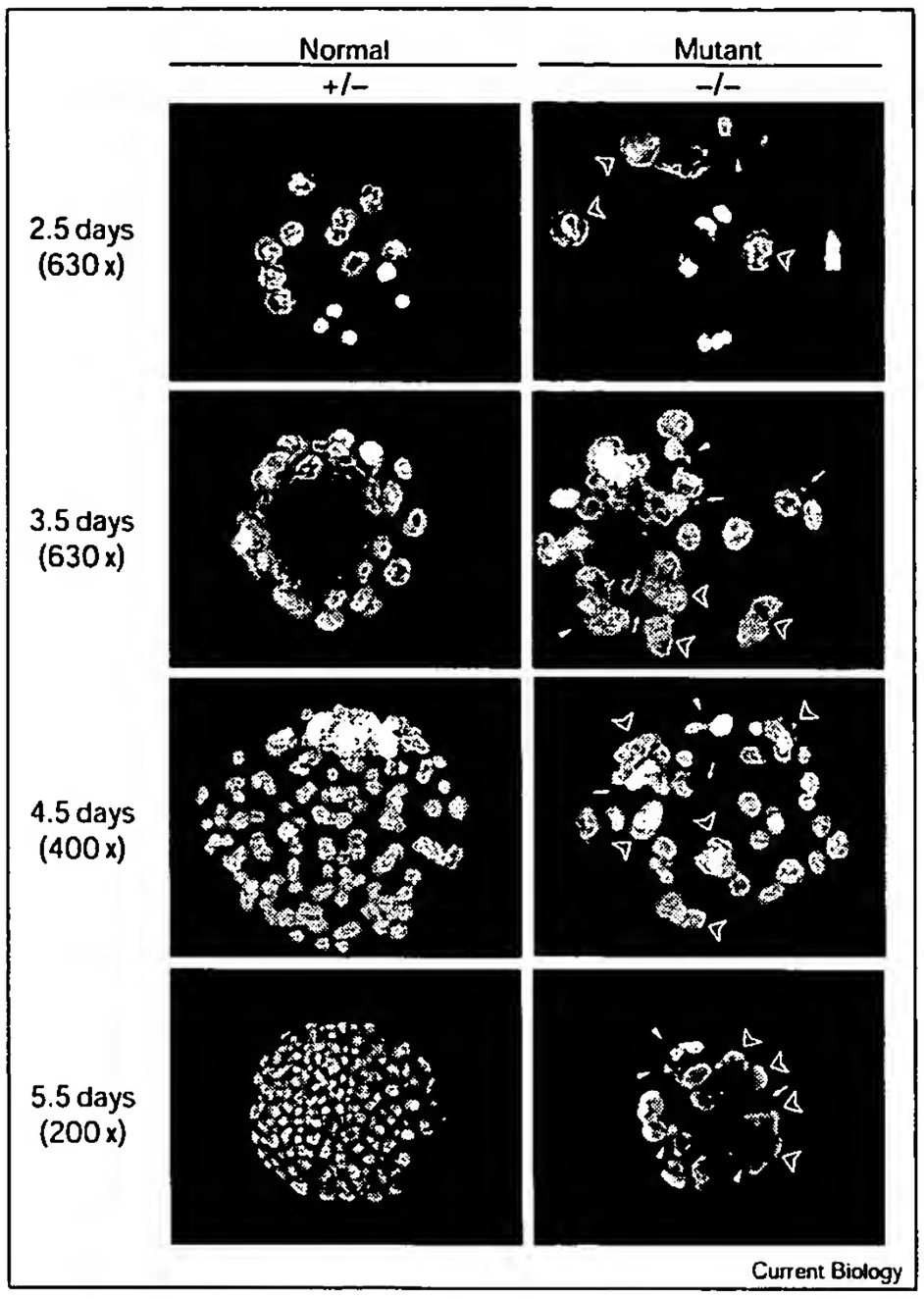
Formation of giant nuclei in *survivin* null embryos

Day 2.5 *post coitum* embryos from heterozygous intercrosses were collected and either analyzed immediately or cultured for up to 3 days before fixing on slides and staining with DAPI so that the number of nuclei and their morphology could be ascertained. At day 2.5, the number of nuclei in the *survivin*$^{-/-}$ embryos was marginally fewer than those in the *survivin*$^{+/+}$ or *survivin*$^{+/-}$ embryos (Table 1). Nuclear morphology appeared normal in some of the *survivin*$^{-/-}$ embryos, but in most the nuclei were irregular and varied in size (Figure 5). At day 3.5, the *survivin*$^{-/-}$ embryos contained only about half as many nuclei as the *survivin*$^{+/-}$ and *survivin*$^{+/+}$ embryos, and micronuclei, irregular nuclei, and some large nuclei were apparent. Day 4.5 *survivin*$^{-/-}$ embryos contained on average less than a third of the number of nuclei found in the corresponding *survivin*$^{+/+}$ or *survivin*$^{+/-}$ embryos. Micronuclei and irregular macronuclei with bridging or lobular morphology indicative of incomplete nuclear fission occurred. By day 5.5, an average of only 13 nuclei were present in each *survivin*$^{-/-}$ embryo, compared with more than 200 in the *survivin*$^{+/+}$ or *survivin*$^{+/-}$ embryos. Most of these nuclei were much bigger than normal, and had highly irregular morphology, with pronounced bridging and blebbing.

Survivin deficiency leads to altered microtubule organization

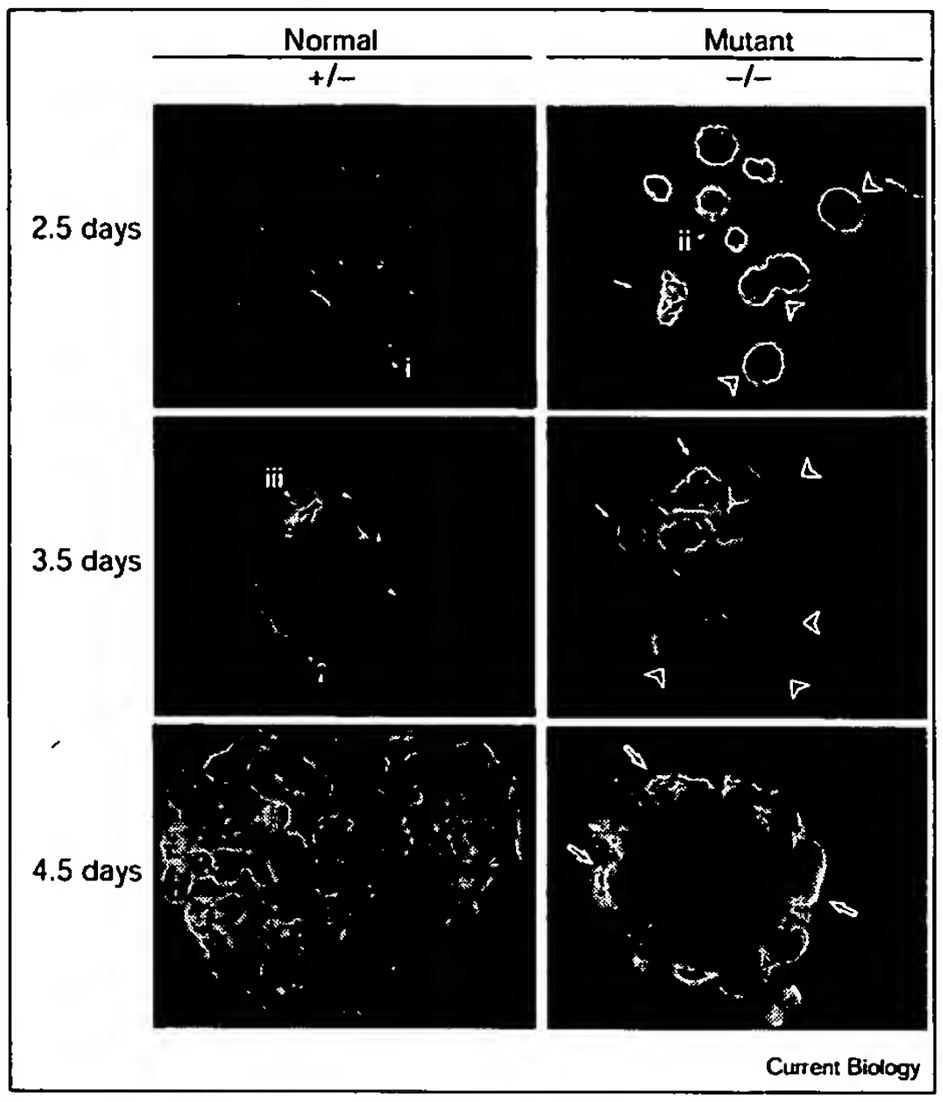
Embryos at different stages of development were stained with anti-β-tubulin antibody to visualize the integrity of the microtubules (Figure 6). Normal embryos showed the expected mitotic spindle structures, midbodies and extensive network of cellular microtubules at all stages. From day 2.5 onwards, *survivin*$^{-/-}$ embryos consistently showed very little or no detectable mitotic spindle organization and midbodies. As the *survivin*$^{-/-}$ phenotype developed, an increasing number of binucleated and multinucleated cells, as well as cells with grossly enlarged and morphologically irregular nuclei were observed. The extensive and fibrous network of tubulin staining seen in normal embryos was progressively replaced by a more diffuse and patchy staining, with an increasing propensity for the tubulin molecules to bundle into highly concentrated strands in the later stages of development.

Figure 5



Nuclear morphology of DAPI-stained embryos cultured at day 2.5 post coitum. Examples of micronuclei (arrows), macronuclei (open arrowheads) and nuclei showing bridging or blebbing (filled arrowheads) are shown. The numbers in brackets indicate the different magnifications used.

Figure 6



Immunofluorescence analysis of tubulin localization in embryos at different developmental stages. In the normal embryo at day 2.5, a dividing cell (denoted i) is seen at the telophase stage, showing a well-organized midbody, whereas no discernible midbodies were detected in the *survivin*$^{-/-}$ embryos at day 2.5 (denoted ii). At day 3.5, the normal dividing cell (denoted by iii) shows a proper microtubule spindle, but this was not seen in *survivin*$^{-/-}$ embryos at day 3.5. Examples are shown of midbodies in the day 2.5 and 3.5 normal embryos (solid arrowheads; not shown for the day 4.5 mutant embryo as most of the nuclei were large), and binucleated or multinucleated cells (solid arrow), and highly bundled microtubule spindle cords in the day 4.5 mutant embryo (open arrows). Magnification ×630.

Discussion

Survivin is a new member of the family of centromere-binding passenger proteins

In agreement with earlier studies showing expression of this gene in the G2/M phase of the cell cycle [25,26], immunofluorescence analysis showed that Survivin is undetectable at interphase but first appears in the nucleus in early prophase. Maximal expression was seen in late prophase, during which the protein was present throughout the nucleus. Binding to the centromere first occurred at this stage and strengthened into metaphase, with a concomitant reduction in the intensity of the nuclear staining, suggesting a relocation of Survivin onto the maturing centromere. As the sister chromatids moved apart during anaphase, Survivin dissociated from the centromere and tethered at the spindle midzone, where it subsequently formed a midbody to be degraded at telophase. Thus, the cell-cycle distribution pattern for Survivin corresponded to those of the chromosome passenger proteins (reviewed in [16,35]). Previous studies have suggested that the distribution of Survivin is closely associated with microtubules [26,36]. Treatment of cells with three different microtubule-inhibiting agents (taxol, nocodazole and colcemid), or mechanical disruption of microtubules by cytocentrifugation, had no major effect on the centromere localization of Survivin. This observation suggests that the cell-cycle distribution of Survivin, in particular its relocation to the centromere during metaphase, is not dependent on the integrity of the mitotic microtubules.

Survivin and INCENP bind to the centromere on a novel para-polar axis

Immunofluorescence showed the two constitutive centromere proteins CENP-A and CENP-B, as well as two of the chromosome passenger proteins CENP-E and CENP-F,

to bind along the trans-polar axis containing the kinetochore plates. Along this axis, CENP-E and CENP-F bound external to the positions occupied by CENP-A and CENP-B. These results are consistent with those of previous studies demonstrating localization of CENP-A, CENP-B, and CENP-E/CENP-F at the kinetochore domain, inner centromere domain, and fibrous corona domain, respectively [37–40].

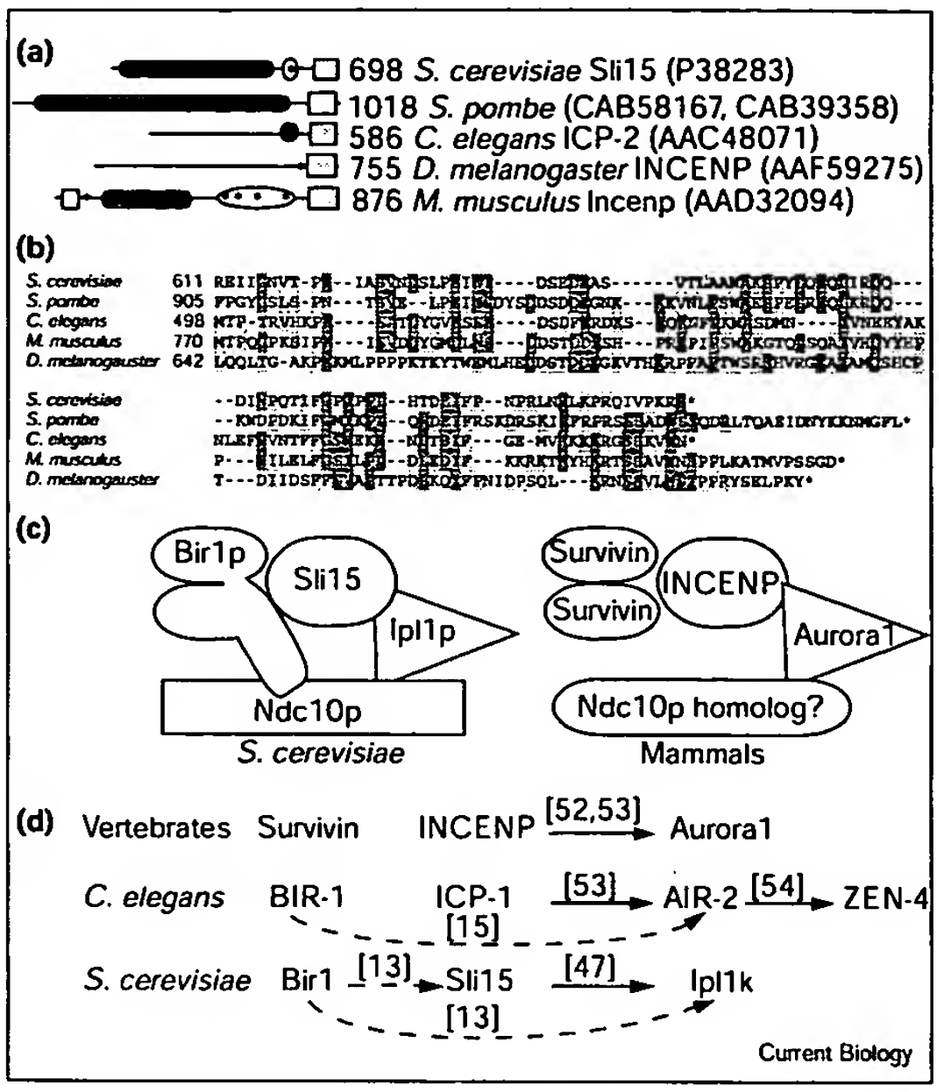
In chicken cells INCENP has been localized either as a doublet between the sister chromatids on an axis parallel to the longitudinal axis of the chromosome (y-axis; Figure 2g) [41], or throughout the heterochromatin beneath the kinetochore (Figure 2g) [42,43]. In contrast, in human HeLa cells we found both INCENP and Survivin bound along a para-polar z-axis that perpendicularly bisects the longitudinal y-axis and the trans-polar x-axis. The reason for the discrepancy is unclear, but could be related to differences between vertebrate classes, or the sensitivity and resolution of the techniques used.

The pattern of localization we observed suggests that the chromosome passenger class of centromere proteins can be subdivided into two subgroups depending on whether they reside along the trans-polar axis or the para-polar axis. The trans-polar orientation would allow the organization of the structural proteins (and their interacting DNA) into configurations (the kinetochore discs) such that their active faces will point toward the spindle poles to provide opposite attachment sites for microtubules. This orientation would also facilitate the functioning of proteins, such as the molecular motor CENP-E [44,45], whose activity may depend on direct, end-on interactions with the kinetochore-associated microtubules to effect chromosome movement [46]. The para-polar orientation would favor roles such as sister chromatid cohesion, side-on centromere interaction with microtubules, or provides a depot to facilitate the relocation of proteins onto microtubules for downstream functions, such as regulation of microtubule activity and cell cleavage.

Survivin disruption results in microtubule bundling and impaired cytokinesis resembling that of INCENP null mice

The onset of an abnormal phenotype with *survivin* gene disruption varied slightly amongst the embryos, with some embryos exhibiting normal morphology until day 4.5 *post coitum*, while others showed deterioration as early as day 2.5. Irrespective of the time of onset, all null embryos became grossly affected by day 5.5. Early signs of embryo deterioration included degenerating blastomeres, micronuclei formation, variable nuclear sizes, irregular nuclear morphology and multinucleation. These aberrations were characteristic of an underlying defect in mitosis. As the phenotype progressed, cells ceased to complete mitosis, with the decreasing number of normal cells rapidly replaced by a small number of giant cells with large and morphologically unusual nuclei. Tubulin staining revealed the absence of normal mitotic spindle structures and intercellular midbodies, with reduced microtubule networks around the cells, and bundling of microtubules. Thus, the observed phenotype was very similar to that previously described for the INCENP null mouse embryos [22]. In both cases, the phenotype was consistent with a defect affecting microtubule organization and/or cytokinesis.

Figure 7



Like Survivin and Bir1, INCENP and Sli15 appear to be homologs with conserved functions. **(a)** Proteins bearing an INCENP box (light gray box) can be found in organisms ranging from yeasts to vertebrates. Asterisks, putative nuclear localization signals; dark gray ovals, regions resembling neurofilaments; white box, a region of INCENP shared by vertebrate homologs and required for centromere targeting [23]; white ovals, predicted coiled-coil domains of INCENP and Sli15. The respective sequence database accession numbers are indicated in parentheses. **(b)** Comparison of INCENP box sequences, with universally conserved residues highlighted in blue and less well-conserved residues in shades of gray. **(c)** Relationship of a BIR-containing protein (Bir1p), INCENP protein (Sli15) and Aurora homolog (Ipl1p) to the centromere-binding protein Ndc10p in *S. cerevisiae* is shown on the left. Bir1p binds to Ndc10p through its carboxy-terminal half, rather than through its BIRs, which are in the amino-terminal region. Hypothetical relationship of the corresponding homologous mammalian proteins (Survivin, INCENP and Aurora1) is shown on the right. **(d)** Survivin, INCENP and Aurora1 and their homologs have been observed to interact with each other directly or indirectly in a hierarchy that often determines their localization. Arrows with reference numbers indicate interactions that have been shown to be direct (solid arrows) or may be indirect (dashed arrows).

Relationship between Survivin, INCENP, Aurora kinase and Ndc10p

A number of observations suggest that Survivin and INCENP may have related roles in mitotic cell division. Both are members of the chromosome passenger class of proteins with very similar cell-cycle expression, distribution pattern and gene knockout phenotype ([22,42] and this study). On metaphase chromosomes, Survivin and INCENP both bind to the para-polar axis of the centromere, as distinct from binding along the trans-polar axis that typifies most of the other centromere proteins. Furthermore, the functions of these proteins appear to be highly conserved, because yeasts have homologs of both Survivin [2] and INCENP (Figure 7a,b) that are also involved in chromosome segregation. Mutation of Bir1p, the closest homolog of Survivin in *S. cerevisiae*, causes a chromosome-loss phenotype [13] and, although homologs of INCENP have not yet been described in invertebrates, proteins that share a similar carboxy-terminal motif to that of INCENP, here dubbed the 'INCENP box', can be found in the mouse, *Drosophila*, *C. elegans*, *S. pombe* and *S. cerevisiae* (Figure 7a,b). Significantly, there is a single INCENP-box-bearing protein in *S. cerevisiae*, termed Sli15, which, like INCENP, associates with the spindle and is required for proper chromosome segregation [47].

It is interesting to note that the *S. cerevisiae* homologs of both proteins interact directly or indirectly with the same protein, the serine threonine kinase Ipl1p. Thus, Sli15 binds and may regulate Ipl1p in yeast [47], and Bir1p binds the Ipl1p substrate Ndc10p, a key component of the *S. cerevisiae* kinetochore [13,48,49]. Furthermore, there is also a functional correlation, because mutation of IPL1, like SLI15 and BIR1, causes chromosome missegregation [50]. The association of these proteins is likely to be evolutionarily conserved because interference with *bir-1* in *C. elegans* causes defects in cytokinesis similar to those in which *air-2*, the gene for an Ipl1p-like kinase, is inhibited [11,51], and AIR-2 does not localize to centromeres in the absence of BIR-1 [15].

The mammalian homologs of Ipl1p are the Aurora kinases (reviewed in [20]). Intriguingly, expression of mutant proteins and immunohistochemistry has shown that, in mammalian cells, Aurora1 and INCENP demonstrate similar cell-cycle distribution profiles and have related roles in mitosis [19,21–23]. Thus, it appears that Survivin, INCENP and Aurora1 are part of a mechanism governing chromosome segregation and cytokinesis that has been conserved from the yeasts to mammals (Figure 7c). This model is strongly supported by evidence in papers made available to us after submission of this manuscript showing that INCENP and Aurora1 bind to each other directly, and INCENP is required for Aurora1 localization to centromeres and the central spindle [52]. Furthermore, in *C. elegans*, the INCENP homolog ICP-1 is able to bind the Aurora1 homolog AIR-2 [53]. AIR-2 is in turn required for localization of the kinesin-like protein ZEN-4/CeMKLP1 to the spindle midzone [54]. In *C. elegans*, AIR-2 requires the Survivin homolog BIR-1 for its localization [15], but it remains to be seen whether in mammals (or *C. elegans*) Survivin interacts directly with INCENP or Aurora1, or indirectly, as in the yeast *S. cerevisiae* (Figure 7d).

Two groups of BIR-bearing proteins

Structural and functional considerations suggest that there are two classes of BIR-bearing proteins. Members of one class, which includes Survivin, are primarily involved in cell division, and members of the other are primarily involved in the control of apoptosis. The yeasts and *C. elegans* appear to encode only the former class, whereas other organisms such as *Drosophila* and mammals have proteins of both classes. Perhaps, during metazoan evolution, there was duplication of the gene encoding the primordial BIR-containing protein, which functions in cell division, and because this protein had an intrinsic affinity for caspases the second class of specifically anti-apoptotic BIR-containing proteins developed.

It has previously been shown that expression of Survivin is higher in cancers than in normal tissues [24]. Expression of Survivin during mitosis may explain this correlation as tumor cells have a higher mitotic index than cells in normal adult tissues. It has been proposed that Survivin may contribute to oncogenesis through inhibition of apoptosis, but the cell-cycle role of Survivin raises several additional possibilities. Inappropriate Survivin expression may induce chromosome instability, leading to oncogenic changes in ploidy. Alternatively, Survivin expression may induce inappropriate proliferation in cancer cells, or may simply be required for quiescent cells to escape their normal proliferative restraints. Whatever the function of Survivin in tumor cells, the absence of its expression in most normal adult tissues warrants further investigation of this protein as a target for novel anti-cancer therapeutics.

Conclusions

In *S. cerevisiae*, the proteins Bir1, Sli15 and Ipl1p interact with each other either directly or through the kinetochore protein Ndc10p. Furthermore, yeast mutant for these genes display similar defects in chromosome segregation and cytokinesis. The mammalian homologs of these proteins — Survivin, INCENP and Aurora1 — have similar expression patterns and localization. These observations, and the similar phenotypes of mouse embryos lacking Survivin or INCENP, suggest that the primary role of these proteins is to regulate chromosome segregation and cytokinesis, and they are likely to function in concert with the kinase Aurora1.

Materials and methods

Gene targeting and genotyping of mice

A mouse *survivin* cDNA clone was used to screen a mouse genomic library (strain 129Sv). Multiple independent lambda phage clones

were isolated and mapped by restriction-enzyme digestion; 9 kb of the longest clone was sequenced, encompassing the region encoding the entirety of the mouse *survivin* cDNAs. The targeting vector for *survivin* was electroporated into Bruce 4 (C57Bl/6) ES cells [55], which were then selected in G418-containing medium. Genomic DNA isolated from G418-resistant colonies was digested with *Bam*HI and analyzed by Southern hybridization using a PCR-generated 250 bp fragment 3′ of the homologous regions as a probe. Chimeric mice were generated from a correctly targeted mutant ES cell line (as described in [56]), and high-percentage chimeras were bred to C57Bl/6, and offspring from these crosses were genotyped by Southern hybridization to confirm germ-line transmission of the targeted allele. Mice were maintained on a C57Bl/6 background. Subsequent genotyping of mice was performed by PCR using the following primers: 5′ wild-type primer, 5′-GCAAAGGAGACCAACAACAAGC-3′; 5′ knockout primer, 5′-GGATTAGATAAATGCCTGCTCT-3′; 3′ primer, 5′-CAGCTCTG-CATCATTTAGTGCA-3′. These primers gave products of 0.9 kb for the wild-type allele and 0.6 kb for the mutant allele. Embryos were genotyped by a nested PCR strategy using the above primers for first round reactions and the following primers in the second round of PCR: 5′ wild-type primer, 5′-GGACCTGAGTGACATGCCAC-3′; 5′ knockout primer, 5′-GGCCAGCTCATTCCTCCCA-3′; 3′ primer, 5′-GGTCCTCCTCAATGCAATCAA-3′. The second-round primers gave products of 0.8 kb for the wild-type allele and 0.5 kb for the mutant allele. The reactions contained 1× PCR reaction buffer with $MgCl_2$ (Boehringer Mannheim), 800 µM dNTPs, 800 nM each primer and Taq polymerase (Boehringer Mannheim).

Embryo harvesting, culturing and morphological studies

Mouse breeding pairs were monitored daily for vaginal plugs (day 0.5 of embryonic development). Plugged mice were culled at day 2.5 or day 3.5 post coitum. The uterus and oviducts were dissected and flushed with M2 media. The embryos obtained were used for direct morphological studies, culturing and/or PCR genotyping. For direct morphological studies, day 2.5 and day 3.5 embryos were placed in M16 media (Sigma) under oil, transferred to microwells containing PHEM buffer (45 mM Pipes, 45 mM Hepes, both adjusted to pH 6.7, 10 mM EGTA, 5 mM $MgCl_2$, 1 mM PMSF, 0.7% Triton X-100) for 4–8 min at 37°C, before the embryos were placed individually on glass slides and fixed in a droplet of methanol. After two rinses in fixative, the embryos were stained and mounted in Vectashield antifade mounting medium (Vector Laboratories) containing 20 µg/ml DAPI. For the morphological study of days 4.5, 5.5 and 6.5 embryos, embryos were cultured on gelatinized (0.1% gelatin in PBS) coverslips (22 mm × 22 mm) in 35 mm Petri dishes (Nunc) with ES cell media supplemented with LIF and β-mercaptoethanol at 37°C, 5% CO_2 and photographed daily. The embryos were harvested by rinsing in PBS and treating with 0.25% trypsin for 3–5 min to detach the trophectoderm cells. Microglass pipettes were used to collect the cells. Harvested embryos were used for PCR genotyping or fixed in methanol/acetic acid for 10 min followed by staining as above and analysis on an Olympus 1X70 microscope/Nikon F-601 camera.

Immunofluorescence analysis

HeLa cells used for immunofluorescence analysis were either harvested and cytospun onto a slide or were cultured directly on a coverslip. Cytospun cells or cells grown on coverslips were fixed and processed for immunocytochemistry as described previously [57]. In some experiments, cells were treated with microtubule-inhibiting drugs before harvesting as follows: Taxol (PacliTaxel; Sigma) or nocodazole (Sigma) was added to the culture at 10 µM concentration for 2 h at 37°C. Colcemid (Gibco) was used at 0.1 µM for 1 h at 37°C. Immunohistochemistry was performed essentially as described previously [22]. The rabbit anti-Survivin antibody TO65 was raised against a peptide (sequence APTLPPAWQPFLKDHRI) derived from residues 3–19 at the amino terminus of human Survivin. The peptide was derivatized with an amino-terminal cysteine residue, coupled to keyhole limpet hemocyanin and rabbits were immunized five times (Alpha Diagnostics). The anti-Survivin antibody was affinity purified on the immunizing peptide on a SulfoLink column (Pierce) with elution in 100 mM glycine pH 2.5, according to manufacturer's instructions. The titer and specificity of the antibody was determined and confirmed by ELISA against the immunizing peptide and western blotting of Survivin-transfected cells, which clearly demonstrated specific immunostaining of the Survivin band (data not shown). CREST#6 was an autoimmune serum that detected CENP-A and CENP-B [57]. CENP-B detection used a monoclonal antibody 2D-7 [58]. Rabbit anti-chicken INCENP antibody was the generous gift of W.C. Earnshaw (University of Edinburgh, UK), and rabbit anti-CENP-E and anti-CENP-F antibodies were kindly provided by T.J. Yen (Fox Chase Cancer Center, Philadelphia). Mouse monoclonal anti-β-tubulin antibody (Boehringer) was used diluted 1:25 in PBS containing 3 mg/ml BSA (PBS-BSA).

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